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14. ABSTRACT
Assemblies that sequester guest molecules based on its solubility are of immense interest to carry out extraction as well as reactions particularly when molecules have distinct solubility profiles. The U.S. Army has an interest in this area due to the possibility of using the assemblies to decontaminate and destruct the chemical warfare (CW) agents. Amphiphilic polymer based assemblies that differentially sequester guest molecules are potential candidates towards neutralizing the CW agents. It is also important to modify these assemblies in such a way to disassemble in

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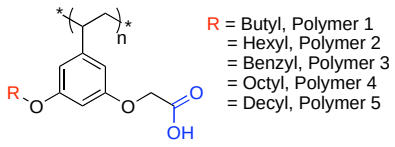
(i) Motivation and key features

Assemblies that sequester guest molecules based on its solubility are of immense interest to carry out extraction as well as reactions particularly when molecules have distinct solubility profiles. The U.S. Army has an interest in this area due to the possibility of using the assemblies to decontaminate and destruct the chemical warfare (CW) agents. Amphiphilic polymer based assemblies that differentially sequester guest molecules are potential candidates towards neutralizing the CW agents. It is also important to modify these assemblies in such a way to disassemble in response to stimuli. Such assemblies are useful in areas such as targeted delivery in addition to neutralizing the CW agents. Towards this goal, we synthesized polymers that could form stable assemblies in heterogeneous solvent mixtures. These assemblies exhibit nanocontainer properties by extracting molecules from both aqueous and organic solvents. We also demonstrated that the disassembly of these nanocontainers could be triggered by chemical and biological stimuli. Certain environments have more than one stimulus, which could be considered as a marker for a specific disease. For targeted delivery in such environments, the nanoconatiner should poses functionalities that respond to multiple stimuli. Towards this goal, we synthesized block copolymers that respond to pH, redox and temperature. Thus the key features of this report are

(i) Synthesis of homopolymers that could form both micelle and reverse micelle type assemblies depending on the nature of the solvent
(ii) Investigation on the nanocontainer property of these assemblies and dye extraction studies to probe the stability of them in heterogeneous solvent mixtures
(iii) Disassembly of organized assembly triggered by redox stimulus
(iv) Disassembly of the nanocontainers using pH, redox and temperature stimuli

(ii) Synthesis and characterizations of the polymeric material

A series of amphiphilic homopolymers (Scheme 1) were synthesized using free radical polymerization method and were characterized utilizing spectroscopic and chromatographic tools. These polymers were found to form micellar and reverse-micellar type assemblies in water and toluene respectively as shown in Figure 2. They were characterized by UV-Visible and fluorescence spectroscopy, dynamic light scattering, and microscopic methods. The versatility of these micellar assemblies was evident from effective encapsulation of several dye molecules in water. In Figure 3 the absorption spectra of one such hydrophobic guest molecule, Reichardt's Dye (RD), in aqueous



Scheme 1: Structure of the polymers

solution of polymer 5 is shown. It can be seen that significant amount of RD is dissolved in water in presence of the polymer micelle. In the absence of polymer micelle the solubility of the dye in water is negligible. Similarly in a non-polar solvent like toluene the reverse micellar type assembly is formed and those can dissolve hydrophilic guest molecules such as Rhodamine 6G (R6G) in toluene, which is not soluble otherwise. The concentrations at which such assemblies are formed were found to be very low (less than ~10^{-6} M) which could be tuned by varying the length of the hydrophobic segment in the monomer unit (Scheme 1).

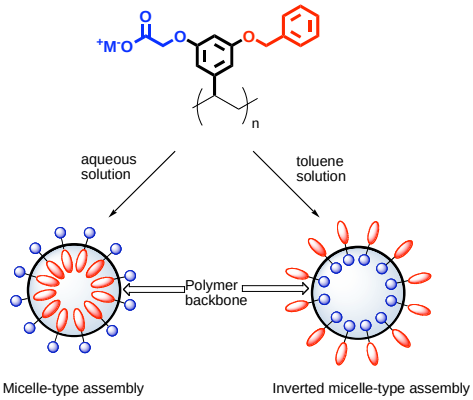


Figure 2: Schematic representation for formation of both micelle and reverse micelle

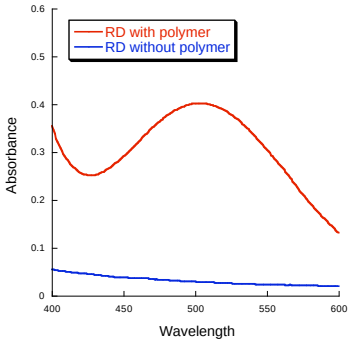


Figure3: Absorption spectrum of RD with in aqueous solution of polymer **5**

(iii) Stability of the assemblies in heterogeneous solvent mixtures

The stability of any supramolecular assembly is an important parameter and micellar assembly is not an exception. The homopolymer system that we have described above present their functionalities in a solvent-dependent fashion that is reminiscent of micelles in water and inverted micelles in an apolar solvent. We asked the question that "what will be the behavior of these micelles in heterogeneous solvent mixtures?" Would these assemblies disintegrate at the interface and find a thermodynamically preferable solvent or would these be stable in the solvents they are initially assembled in? To distinguish these possibilities, we carried out a simple set of experiments. The micellar assembly of polymer **5** (Scheme 1) was formed in water and then mixed with toluene. The resultant heterogeneous mixture was shaken for a while and then allowed to phase separate. The two layers were analyzed for the presence of the polymer. The polymer was completely retained in the water layer and no measurable amount of polymer could be

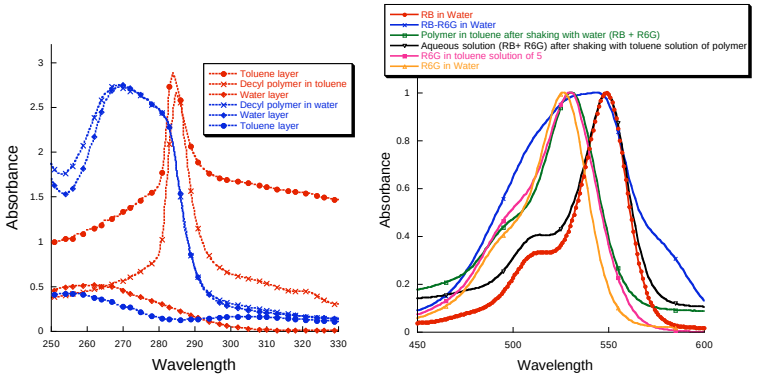
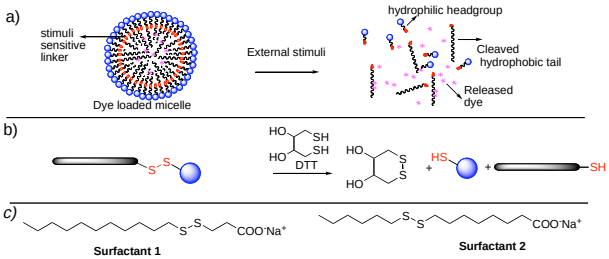


Figure 4: left- The fate of the polymer **5** in heterogeneous solvent mixture; right- selective dye extraction by reverse micellar

seen in the toluene layer (Figure 4). When a similar experiment was carried out with the inverted micelle-type assembly in toluene, the polymer was fully retained in the toluene layer, as shown in Figure 4. Considering this behavior of the polymers in an immiscible mixture of solvents, the possibility of using these assemblies in separations based on electrostatics-based host-guest interactions exists. Accordingly, a toluene solution of polymer **5** was added to an aqueous solution containing a mixture of R6G (cationic in nature) and RB (anionic in nature). After shaking the two solutions for a short time, both layers became colored. Analyzing the toluene layer and aqueous layer revealed that the polymer **5** was able to selectively extract R6G out of the aqueous layer, while leaving behind RB in water (Figure 4). The selectivity we believe is achieved based on electrostatic interaction between the polymer micelle and the dye molecule. From the absorption spectra, the separation seems to be quantitative. These findings imply that it's possible, in principle, to carry out a reaction inside this polymeric nanocontainers followed by selective extraction of the product, which is conceptually an important step for the motivations of this Army-sponsored project.

(iv) Redox sensitive disassembly of organized assembly

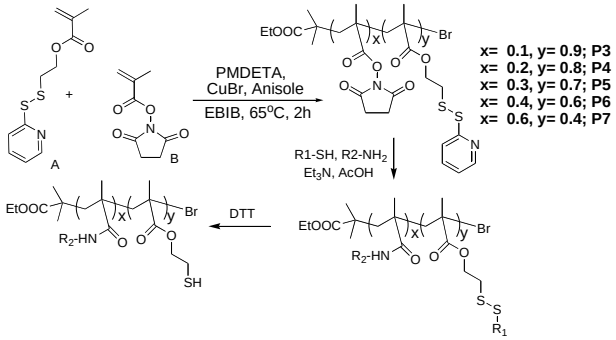
So far, we have demonstrated that amphiphilic polymer based nanocontainers are potential candidates for decontaminating CW. While decontaminating the warfare agent is important, it is also equally important to find out whether disintegrating the container that is used for decontamination is possible under mild conditions. If such disintegration causes release of the load in the nanocontainer in a selected environment then that is interesting to other fields such as targeted delivery. While addressing such fundamental questions, it is useful to test whether the placement of the cleavable linker in different positions is affecting the disassembly process. To examine such possibility we have chosen small molecule surfactant as a model system to precisely vary the placement of the cleavable linker. We chose disulfide as cleavable linker due to the facile cleavage of disulfide bond under very mild reducing conditions. The disulfide linker is placed in between the hydrophilic head and hydrophobic tail of the molecule for surfactant **1**, but placed near the hydrophobic tail end for surfactant **2** (Scheme 2). Surfactant **1** in a mmol concentration makes organized assembly that encapsulates various hydrophobic dye molecules such as pyrene, nile red etc. But upon adding DTT or glutathione it releases the dye molecules and the surfactant is cleaved into water-soluble part and insoluble part (Scheme 2). When we tested surfactant **2**, under similar reducing condition we got identical results to that of surfactant **1**, which indicates that the placement of disulfide linker at different position may not affect the disassembly process.



Scheme 2. **a**) Schematic representation of stimuli-responsive micellar disassembly and guest release; **b**) conversion of amphiphilic surfactant to hydrophilic and lipophilic components in the presence of DTT; and **c**) structures of **Surfactant 1** and **2**.

Synthesis of Polymers with Cleavable and Modifiable Functionalities

In the preceding section, we have shown that small molecule amphiphiles functionalized with disulfide moiety is capable of forming nanocontainers that disassemble to redox stimulus. Due to the versatility provided by the polymers, we have extended our work towards cleavable polymeric amphiphiles. In order to achieve that we have synthesized a copolymer starting from two reactive monomers which are complementary to thiol and amine functionality. (monomer A and B respectively in Scheme 3). In the polymer, succinimidyl ester can be cleaved to form carboxylic acid group that would render amphiphilicity to the polymer. However, a reducing agent such as DTT would cleave the disulfide moiety and convert the amphiphilic polymer to a hydrophilic polymer. Such a conversion would lead to the disassembly of the organized assembly. In addition to the disassembly, the functionalities in this polymer (Scheme 3) provide an interesting opportunity for us to carry out modifications at will. For example, we have demonstrated that these copolymers can be simultaneously functionalized in one pot with an amine and a thiol containing molecules in almost quantitative yield. More interestingly, one of these two connectivities, i.e. the disulfide bond is reversible and cleaved in reducing environment. This has been demonstrated by fluorescence anisotropy experiments by attaching a fluorophore containing thiol to the polymer backbone and comparing its anisotropy when in nascent form. The anisotropy value decreased significantly when the polymer was treated with DTT, ensuring the reversibility of the disulfide functional group in our system. We believe these findings will enable us to make the desired material with much easier synthetic pathway whereas the succinimidyl ester can be functionalized with required amount of three different amines containing hydrophobic, hydrophilic and polymerizable groups.



Scheme 3: Copolymerization of two reactive monomers and simultaneous reversible modification

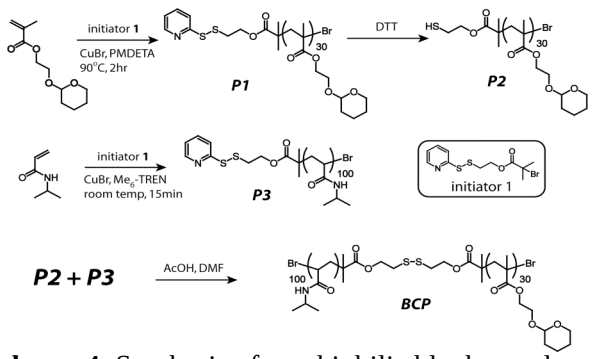
(v) Design and synthesis of multiple stimuli sensitive block copolymer

It is interesting to synthesize polymer that respond to more than one stimulus. For example, the environment around cancer cell is acidic and reductive, hence a nanocontainer that respond to both stimuli is more suitable than a container responsive to one stimulus. Towards this goal, a multiple stimuli sensitive amphiphilic block copolymer was synthesized as shown in Scheme 4. Briefly, a homopolymer **P2** comprising acid sensitive cyclic acetal functionality and homopolymer **P3** comprising temperature sensitive NIPAM was synthesized by controlled radical polymerization. These two homopolymers were then coupled to form a block copolymer **BCP** in presence of AcOH and DMF. The block copolymer formation was confirmed by GPC, which

shows that the molecular weight of block copolymer is the sum of individual homopolymers. The **BCP** formation was also confirmed by spectroscopic techniques.

Investigation of nanocontainer formation

The block copolymer comprises a temperature sensitive hydrophilic block and acid sensitive hydrophobic block, hence it is likely that the block copolymer forms micelle type assembly in water. The assembly formation and container property of the block copolymer was studied using Nile red as a probe. From the emission spectra, we could infer that the interior of the assembly is hydrophobic that results in Nile red encapsulation. We also calculated the critical aggregation concentration (CAC) of the block copolymer, which was found to be 0.1 mg/mL. We used dynamic light scattering to find out the size of the assembly and the size was found to be 90 nm. From these experiments, it is clear that the amphiphilic block copolymer forms nanocontainers that are capable of sequestering hydrophobic guest molecules.



Scheme 4. Synthesis of amphiphilic block copolymer

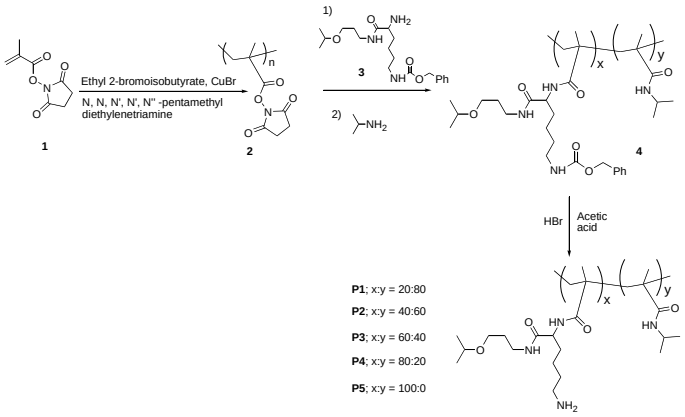
Disassembly of nanocontainers using pH, redox and temperature

The **BCP** has three stimuli sensitive functionalities therefore we anticipate the assembly to disassemble upon exposure to any one of the stimulus. To test this, we exposed the Nile red encapsulated assembly to pH 4 phosphate buffer and monitored the dye release. In two days 60% of the dye molecules were released compared to 20% dye released in pH 6 phosphate buffer. We also observed that the size of the assembly decreased from 90 nm to 30 nm upon exposure to pH4 phosphate buffer. This set of experiments corroborates the disassembly of the micelle type aggregate by cleavage of cyclic acetal moiety in acidic pH. To test the possible disassembly of micelles due to reduction, the Nile red encapsulated assembly was exposed to 5mg/mL of glutathione. The assembly released all the sequestered Nile red molecules in three days, which confirms that the disassembly can be triggered by reduction. The dye encapsulation experiments indicate that the exterior of the aggregate is hydrophilic due to the presence of NIPAM. If that is the case, the desolvation of NIPAM moieties should also result in disassembly of the aggregate. To test this, the assembly in water was heated above 40℃ that resulted in the precipitation of the polymer. This observation reveals that the micelle type assembly disassembles to temperature stimulus as well. From the above experiments, we have demonstrated that the micelle type aggregate can be disassembled in response to multiple stimuli.

(vi) Investigation of amphiphilic polymer in controlling the enzymatic action

So far we have demonstrated as how one can extract molecules and release the encapsulated molecules as function of stimului. The assemblies shown in this report have the potential to impact the Army's efforts in decontaminating the warfare agents. We now explore the possibility of utilizing the amphiphilic polymer based assemblies in destructing the warfare agents.

Enzymes with modified activity and enhanced substrate selectivity are of great interest in biocatalysis. Noncovalent interactions between artificial macromolecular scaffolds and enzymes are further interesting due to the possibility of reversibly modulating the enzyme activity or turning the enzyme reaction "on" or "off" whenever needed. It will be very relevant to Army research office when a modifiable enzyme used for catalyzing a reaction with a chemical warfare agent. As an example, a CW agent can be deactivated using organophosphorus hydrolase (OPH) enzyme and the efficiency of which can be modified using a polymeric scaffold that binds to the enzyme. The hypothesis here is that the polymer might stabilize the enzyme that is favorable for the particular reaction, therefore the overall efficiency and the selectivity of the reaction will be very high. To identify such a possibility we have taken chymotrypsin (ChT) (Figure 5) as a model enzyme and tested its substrate selectivity by interacting it with amphiphilic cationic copolymers. Here, we achieved a simple design that has polymer with minimum hydrophobicity to interact with enzyme but does not form micelle. While micelle forming polymer might interact with certain hydrophobic substrates; we decided to test the non micelle forming polymers. To achieve such polymers we have designed and synthesized polymers **P1**-**P5** (Scheme 5) with varying hydrophobicity and cationic charge. Considering the cationic nature of ChT (pI ~ 8.8), it is unconventional to expect an interaction with cationic polymer, but by cautiously looking at the structure of ChT (Figure 5) one can possibly predict that these polymers will bind to the enzyme (ChT) due to the presence of multiple anionic residues on the surface of the ChT.



Scheme 5. Synthesis of random copolymers **P1**-**P5** with different density of cationic residues

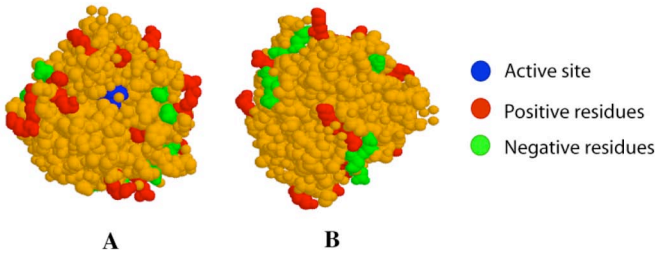


Figure 5. Front (A) and rear (B) view of α-Chymotrypsin.

The polymers **P1**-**P5** (Scheme 5) are synthesized from lysine-based compound **3** as amphiphilic monomer and the charge or hydrophobicity varied by diluting it with isopropyl methacrylamide as neutralizing monomer (Scheme 5). To gain insight into the polymer structure and its properties in enzyme binding, it might be important that the polymer has identical chain length. In order to ascertain such polymer we have made poly(*N*-hydroxy succinimide methacrylate) (M_n= 15.3 kDa, PDI = 1.12 and DPI = 83) an amine functionalizable polymer that was reacted with targeted monomer **3** and isopropyl amine as neutral monomer to required polymer. The relative ratio of these two is varied to get **P1** to **P5** in which the ratio of targeted monomer to neutral monomer is increased as we go from **P1** to **P5** (Scheme 5).

The enzymatic activity (amidolysis) of ChT in the presence of polymers **P1**-**P5** was tested against cationic (S1), neutral (S2) and anionic (S3) substrates. All of these substrates are identical in structure except the charge. The percent enzymatic activities of ChT are shown in Figure 6, which are normalized to the activity of native ChT with particular substrates in the absence of the polymer. The polymer **P1** has 20 % cationic functionality hyperactivates the ChT towards cationic substrate (169 %) and inhibits towards anionic one (29 %). Whereas polymer **P5** that has 100 % cationic residues, hyperactivates the enzyme towards all of the three substrates, and the other polymers **P2**-**P4** lie in between. The entire polymer mediated modulation of ChT turns out to be reversible upon adding 50 mmol of sodium chloride, which also indicates that the noncovalent interaction is mainly based on electrostatics. The circular dichroism and fluorescence spectroscopy studies revealed that these polymers did not denature the enzyme (not shown here).

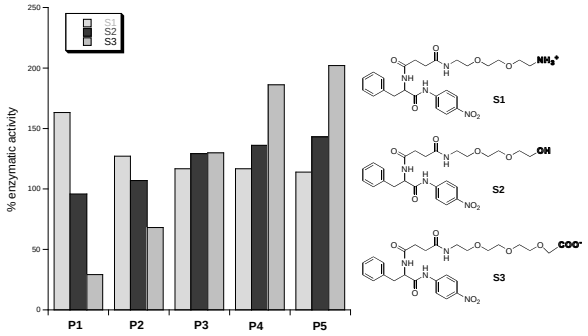


Figure 6. Modulation of enzymatic selectivity under the influence of polymers **P1**-**P5**

This particular evidence illustrates the possibility of using the polymer mediated modulation of enzymatic action is a viable approach to increase the selectivity and efficiency of an enzyme.

(vii) Summary

We have designed and synthesized a new class of amphiphilic homopolymers which shows both micellar and reverse micellar property. These assemblies are stable in heterogeneous solvent mixture and can selectively extract dye molecules based on differential electrostatic interaction. Assemblies formed using small molecule amphiphiles can be disassembled under very mild conditions and we have shown that placement of cleavable linker may not affect the release or disassembly process. We have extended our strategy of incorporating cleavable functionalities to polymers. We have shown that amphiphilic block copolymers comprising multiple stimuli sensitive functionalities can form micelle type assemblies. Further, the nanocontainers can disassemble and release the guest molecules in response to mild stimuli such as temperature, redox and pH. We have also shown that amphiphilic polymer can be used to

increase the efficiency and selectivity of an enzyme to a particular substrate. We strongly believe that these fundamental studies would enable us to access simple material and methods to decontaminate and destruct chemical warfare agents.

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